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PRINCIPAL INVESTIGATOR: Philip S. Zeitler, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado
Health Sciences Center
Denver, Colorado 80045-0508

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Philip S. Zeitler, Ph.D.

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University of Colorado Health Sciences Center
Denver, Colorado 80045-0508
email – Phil.Zeitler@uchsc.edu

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13. ABSTRACT *(Maximum 200 Words)*

. We hypothesize that **GHRH functions as an autocrine/paracrine growth factor in neoplastic breast tissue**. To address this hypothesis, we are undertaking a comprehensive examination of the physiology of GHRH in immortalized breast cancer cell lines. We report here the results of the second 12 months of this project. The data summarized here indicate that endogenous GHRH acts as a growth factor through activation of MAPK/ERK (in a ras and/or raf dependent fashion). In addition, the data suggest an anti-apoptotic action of GHRH through suppression of p38 activation of a caspase cascade (9≻3≻2) and consequent inhibition of Bcl-2 cleavage. Activation of an independent Jnk pathway may antagonize the effects of GHRH on the p38 pathway. The emerging picture of the pathway by which GHRH promotes growth and inhibits apoptosis in breast cancer cell lines furthers our understanding of the previously demonstrated actions of GHRH antagonists to inhibit breast cancer growth *in vitro* and *in vivo*. More importantly, this understanding begins to suggest ways in which GHRH antagonists might fit into therapeutic regimens, as pro-apoptotic agents in their own right or as adjuvant agents supporting the action of traditional anti-neoplastics.

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INTRODUCTION

Growth hormone-releasing hormone (GHRH), in addition to stimulating the release of growth hormone (GH) from the pituitary, acts as a trophic factor for pituitary somatotrophs[1-8]. In addition, GHRH is expressed outside of the hypothalamic/pituitary axis, with significant expression in the gonads, gastrointestinal tract, pancreas, thymus, and lymphocytes[9-14]. Expression has also been reported in a variety of tumors[15], including pancreas, lung, CNS[16] and, recently, breast, prostate, and endometrium[17-19]. While the role of extrahypothalamic GHRH is unknown, mitogenic effects have been demonstrated on lymphocytes and germ cells *in vitro*[20,21]. The current study examines the hypothesis that ***GHRH functions as an autocrine/paracrine growth factor in neoplastic breast tissue.*** To address this hypothesis, we have undertaken a comprehensive examination of the physiology of GHRH in immortalized breast cancer cell lines. We examine the effect on cell growth and proliferation of exogenous GHRH and disruption of endogenous GHRH with specific inhibitors. We also identify the intracellular signaling pathways that mediate the effects of GHRH on breast cancer cells. Finally, we will dissect the mechanism by which GHRH and its receptor begin to be expressed in the process of neoplastic transformation of breast tissue. We report here the results of the second 12 months of this project. During this period, we have expanded the investigations performed during the first 12 months of the project, with particular attention to the participation of the p38 pathway, intracellular caspases, and the pro-survival protein Bcl-2. We have also demonstrated that MAP kinase, through a raf/ras dependent pathway, mediates the growth promoting effects of GHRH in breast cancer cell lines.

BODY

Task 1: To determine the relationship between expression of GHRH, expression of GHRH receptor, and cellular proliferation in breast cancer cell lines (Months 1 - 15)

Quantitate the expression of protein and mRNA for GHRH and GHRH receptor in breast cancer cell lines (Months 1 - 4)

We have continued to be stymied by the technical problems described in the Annual Report of 2001, namely the unavailability of reagents for the approaches originally proposed. In the absence of information regarding expression of these genes in other breast cancer cell lines, work has continued to focus on the physiology of MDA231 cells, which express both GHRH and the GHRH receptor at high levels.

Define the effect of GHRH and its antagonists on the growth, proliferation, and apoptosis of breast cancer cells (Months 4 -10)

Task completed – results described in Progress Report of July 2001. Manuscript is in Press and included in Appendix

Determine whether changing the expression of GHRH and/or GHRH receptor in breast cancer cell lines results in changes in cell growth characteristics (Months 10-15).

We have begun the proposed transfections and are currently in the process of obtaining growth data. No preliminary data is available at the time of this report.
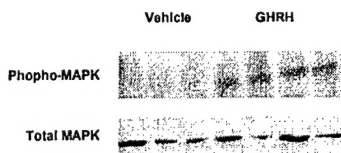
Task 2: To determine the intracellular signaling pathway(s) that mediate the effects of GHRH on growth in breast cancer cells (Months 15 - 20)

Define the generation of cAMP and activated MAP kinase pathway intermediates in breast cancer cells in response to GHRH (Months 15-18)
Determine the pathway(s) that mediates the proliferative and/or apoptotic effects of GHRH (Months 19-24)

The work proposed in this task has continued to progress significantly over the last year, with continued expansion of our understanding of the pathways involved in transduction of the GHRH signal in MDA231 cells. .

Experiments described in the Annual report of 2001 focused on the effects of treating MDA231 cells with GHRH. However, at that time, problems were created by significantly elevated basal levels of ERK 1/2 activation, likely reflecting the actions of endogenous GHRH. At the time of the last report, attempts to decrease basal MAPK activation by manipulation of culture conditions had been unsuccessful. We have subsequently developed a culture paradigm in which culture medium is changed 2 hours prior to obtaining the baseline sample. Under these conditions, basal levels of ERK1/2 activation are markedly suppressed, and rapid phosphorylation of ERK1/2 stimulation by GHRH can be demonstrated.
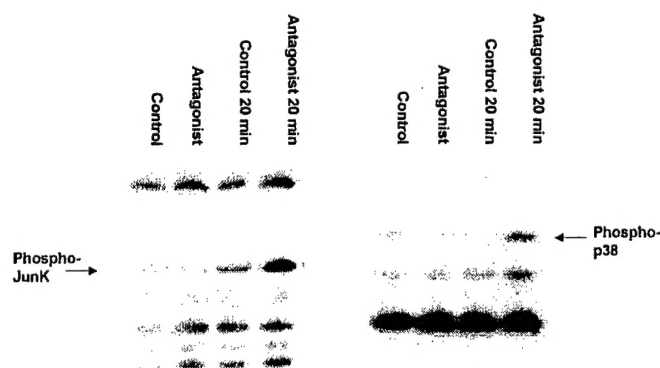

Figure 1: hGHRH (10 nM) incudes MAPK phosphorylation in MDA231 (2.5 minutes)

Thus, the signal transduction pathway for GHRH in MDA231 cells bears a strong similarity to that we have previously described in pituitary somatotrophs[22]. Furthermore, the elevated basal levels of MAPK phosphorylation seen in the absence of removal of endogenous GHRH suggests that endogenous GHRH provides a tonic phosphorylation stimulus to the MAPK pathway under normal conditions. Taken together, the results indicate that endogenous GHRH could play an autocrine/paracrine role in promoting persistent cell growth. We are currently undertaking studies to examine the upstream pathway by which GHRH receptor activation leads to MAPK phosphorylation. Studies using transient transfection with dominant negative *ras* and *raf*, suggest that MAPK activation is dependent on these upstream activators. However, these studies remain preliminary and have not yet been repeated rigorously.
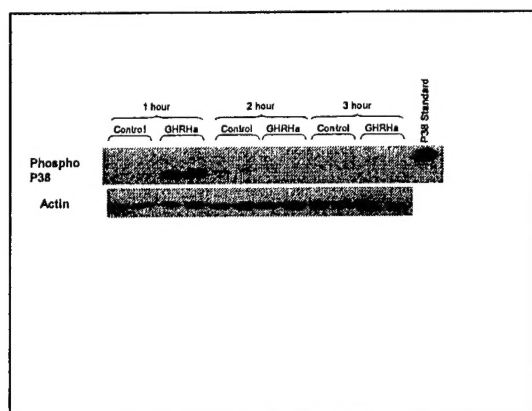
Since antagonism of GHRH leads to increased apoptosis, we have turned our attention to other components of the MAPK pathway known to be related to this phenomenon[23-25]. Activation of the p38 pathway has been associated with initiation of apoptosis in a number of cell systems, while activation of JunK has a more complicated relationship to apoptosis, associated with both stimulation and suppression of apoptosis. Some of this material was included in the Annual Report of 2001, but is included here to provide context for the subsequent studies.

MDA231 cells were exposed to GHRH antagonist (GHRHa) (3 μM) or vehicle for 20 min. Cells were then washed, lysed, and the proteins separated by PAGE and analyzed by Western blot hybridization with phospho-specific Jun kinase or Phospho-specific p38 kinase antibody. As shown in figure 2, exposure to GHRH antagonist resulted in marked activation/phosphorylation of both JunK and p38 kinase compared to vehicle.
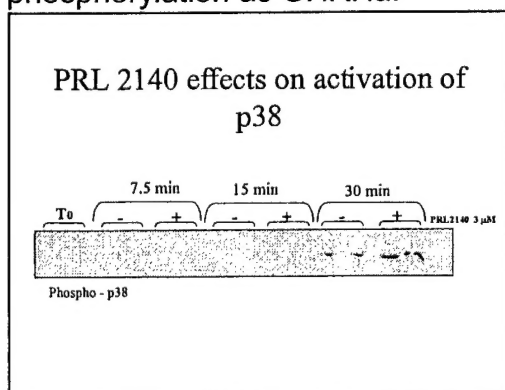

Figure 2: Effect of 3 μM GHRH antagonist on phosphorylation of Jun kinase (left panel) and p38 kinase (right panel) in MDA231 cells.

We next examined the time course of P38 activation in response to GHRHa, using the same experimental paradigm. As shown in figure 3, exposure of MDA231 cells to 3 μM GHRHa leads to dramatic, rapid, and transient phosphorylation/activation of P38, with onset within 20 minutes (Figure 9) and return to baseline before 2 hours. This pattern of P38 activation has been seen in other apoptotic systems.
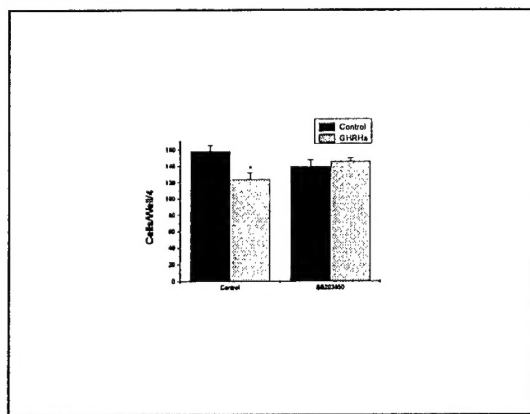

Figure 3: Time course of the effect of 3 μM GHRH antagonist on phosphorylation of p38 kinase in MDA231 cells

To confirm the effect of GHRH antagonism on p38 phosphorylation, we examined the effect of a potent, non-competitive GHRH antagonist, PRL 2140 (David Coy, Tulane University) using the same paradigm. As seen in Figure 4, this independent antagonist shows essentially the same effect on p38 phosphorylation as GHRHa.

Figure 4: Time course of the effect of the GHRH antagonist PRL 2140 on phosphorylation of p38 kinase in MDA231 cells
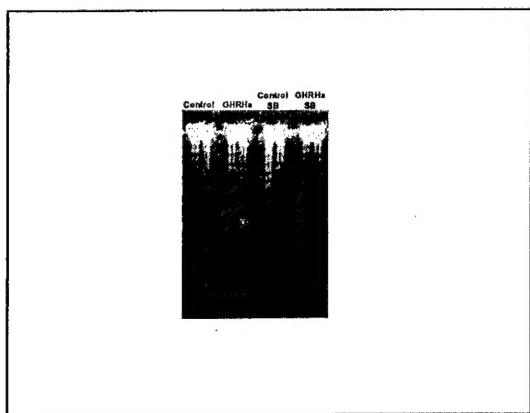
In order to determine whether activation of P38 by GHRHa is related to apopotosis, we examined the effect of the P38 inhibitor SB203580 on cell number reduction and apopotosis in response to treatment with GHRHa. MDA231 cells were pretreated with 10 µM SB203580 30 minutes prior to exposure to GHRHa and cell counting, as described above. As shown in figure 5, inhibition of P38 activation by the SB compound prevented the decrease in cell number seen in response to GHRHa, strongly suggesting that activation of P38 mediates the effect of GHRHa on changes in cell number.

Figure 5: Effect of P38 inhibition on the GHRHa induced decrease in MDA231 cell number.
Each value represents the mean ± SD, n = 8 replicates per treatment. * = P<0.05

We next examined the effect of P38 inhibition on DNA laddering in response to GHRHa treatment of MDA231 cells. Again, cells were pretreated with SB203580 30 minutes prior to exposure to GHRHa and cells processed for DNA laddering as described above. As seen in Figure 6, inhibition of P38 activation by the SB compound prevented the stimulation of DNA laddering seen in response to GHRHa, suggesting that P38 activation is required for the GHRHa stimulation of apopotosis.

Figure 6: Effect of P38 inhibition on the GHRHa induced apoptosis in MDA231 cells

We have now begun to examine downstream components of the p38 pathway leading to apoptosis in response to withdrawal of endogenous GHRH. First, in order to determine the participation of caspases in this process, MDA231 cells were treated with GHRHa (5 uM) as in previous experiments. The lysate was subjected to SDS PAGE Electrophoresis, and transfer to Immobilon-P membrane. After blocking, the 12 kDa caspase-3 small subunit was detected using a caspse-3 specific antibody. As shown in figure 7, the phosphorylation of p38 approximately 1 hour after exposure to GHRHa, seen in previous experiments, is accompanied by a marked increase in activated caspase 3 after approximately 1 hour, followed by a reduction to basal levels.
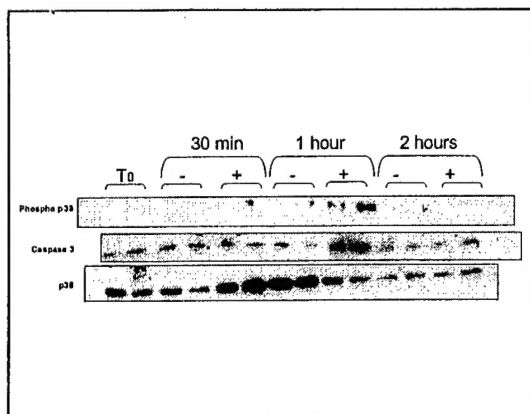
Figure 7: Antagonism of endogenous GHRH with GHRHa (5 uM) promotes p38 phosphorylation and activation of caspase 3.

We have recently obtained the reagents to examine the effect of GHRH antagonism on activation of other members of the caspase family, but cannot yet report the results of these experiments. .

In order to confirm the participation of caspase family members, we examined the effect of caspase inhibition on the cell number reduction following antagonism of endogenous GHRH. MDA231 cells were pretreated with a caspase inhibitors 30 minutes prior to exposure to GHRHa and cell counting, as described above. In this experiment (Figure 8), antagonism of caspases 2, 3, and 9, but not 1,4,6,and 8 prevented the reduction in cell numbers seen 24 hours after exposure to GHRH antagonist, suggesting that activation of caspases, particularly some combination of 2,3 and 9 is required for the effect on apoptosis.
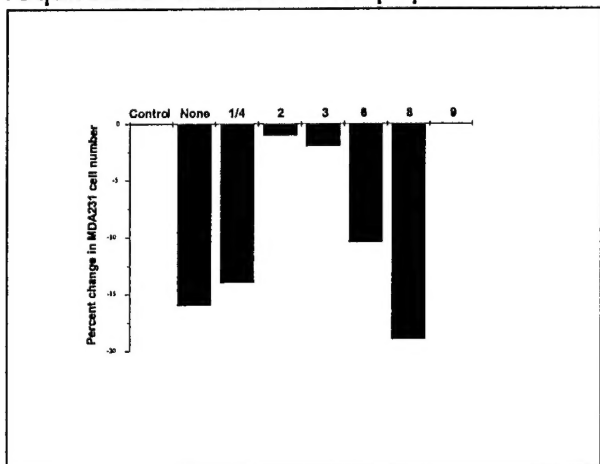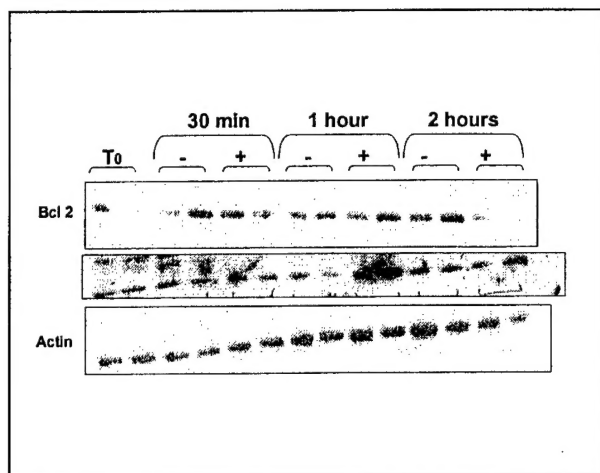

Figure 8: Inhibition of Caspase 2,3 and 9 prevents the GHRHa induced decrease in MDA231 cell number. (n = 4 per group).

Although we have not yet determined the order in which the caspases -2, -3 and -9 are activated, the available literature suggests that caspase-9 is likely to be the initiator caspase in the GHRH antagonized MDA231 cells[26]. In other words, these data suggest that endogenous GHRH in MDA 231 cells blocks a caspase-9 based apoptotic cascade that otherwise would occur. Recently is has been shown that caspase-2 is a direct effector of mitochondrial apoptosis and is inactive towards other caspase zymogens[27]. Similarly, in several human cell lines, apoptosis occurred through an early phase of mitochondrial dysfunction via caspase-2. However, activation of caspase-3 was necessary in these cells for the activation of caspase-2[28]. Thus, since Caspases -2 and -3 are known to be effector caspases, it is reasonable to assume that caspase -2 and -3 act as effectors in our system. *In vitro*, caspase-2 is the preferred cleavage substrate for caspase-3. Thus, it appears that caspase-3 is responsible for the activation of caspase-2 and not *vice versa*[26,28]. Taken together, the data suggest that antagonism of endogenous GHRH leads to activation of a casade from caspase 9 to 3 to 2.

The ratio of pro survival Bcl-2 to pro apoptotic Bcl-2 provides a major regulator of apoptosis[29]. In order to examine the effect of GHRH antagonism on BCL-2 activity, MDA231 cells were treated with
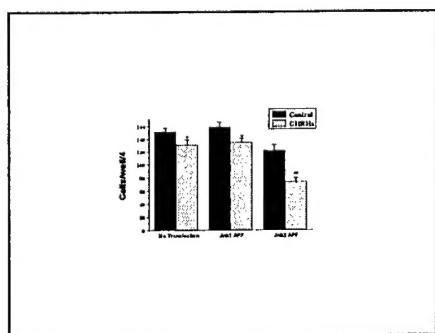
GHRHa (5 uM) as in previous experiments. The lysate was subjected to SDS PAGE and western analysis for the 29 KDa pro-survival BCl-2 protein using Bcl-2-specific antibody. As seen in Figure 9, caspase 3 activation occurs, as previously demonstrated, approximately 1 hour after exposure to GHRHa, followed by disappearance of pro-survival Bcl-2 at 2 hours. Preliminary time course analysis suggests that Bcl-2 remains reduced up to 4 hours after exposure.

Figure 9: Antagonism of endogenous GHRH promotes activation of caspase 3, followed by disappearance of Bcl-2.

Caspase dependent cleavage of pro survival Bcl-2 has been reported. In myeloid leukemic cells, the addition of the caspase-1 inhibitor Z-VAD-FMK prior to treatment with etoposide prevented cleavage of the Bcl-2 protein[30]. Cleavage of Bcl-2 four hours after neocarzinostatin (NCS) treatment of PC-12 pheochromocytoma cells was prevented by the caspase-3 specific inhibitor, Ac-DEVD-CHO[31]. Similarly, removal of IL2, triggers caspase-dependent cleavage of Bcl-2[32-33]. While the experiments described here do not prove that the disappearance of the Bcl-2 protein is due to the activated caspases, the temporal relationship between the two events and the previously described caspase-dependant cleavage of Bcl-2 suggest that the disappearance of Bcl-2 following exposure of cells to GHRHa involves activation of the caspase cascade.

We next examined the effect of inhibition of Jun Kinase on the effect of GHRHa on cell numbers in MDA231 cells. MDA231 cells were transfected with 5 μg of a vector containing a CMV promoter-driven dominant-negative Jun Kinase 1 (Jnk1 APF) or Jun Kinase 2 (Jnk2 APF) construct by electroporation, grown for 24 hours in serum containing medium and then treated with GHRHa as described above. As seen in figure 10, exposure of MDA231 cells to GHRHa in this experiment resulted in a modest (14%) decrease in cell number after 24 hours. Transfection with dominant negative JnK 1 had no effect on this response to GHRHa. However, transfection with dominant negative JnK 2 more than doubled the decrease (39%). Pretreatment of cells with SB203580 completely prevented the decrease in cell number following exposure to GHRHa, even in cells transfected with dominant-negative Jnk 2 (not shown). The amplification of the effect of GHRHa when JnK 2 is inhibited suggests that activation of JnK 2 antagonizes the effect of P38 activation on cell number.
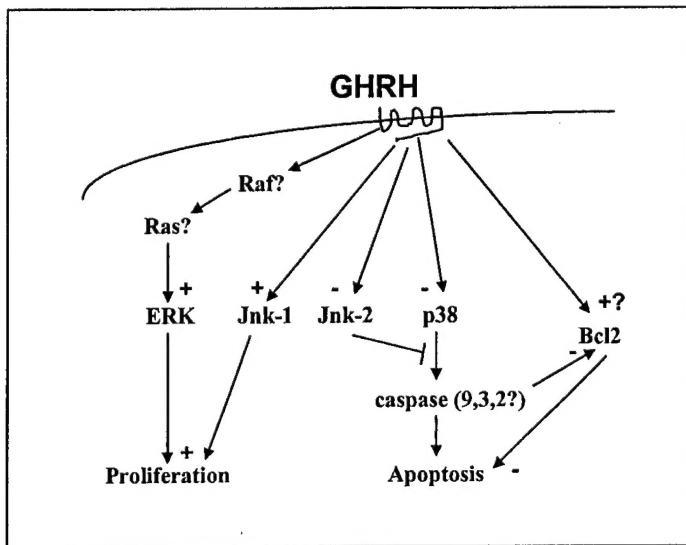

Figure 10: Effect of Jun Kinase 1 and Jun Kinase 2 inhibition on the GHRHa induced decrease in MDA231 cell number.
*Each value represents the mean ± SD, n = 8 replicates per treatment. * = P<0.05, ** = P<0.01*

This experiment suggests that disruption of endogenous GHRH action on MDA231 cells activates both P38 and JunK 2, the former stimulating and the latter inhibiting apoptosis. It is intriguing to hypothesize that the physiologic state of the cell at the time of GHRH disruption influences the relative degree of activation of these two pathways, leading to alterations in the eventual response of cell proliferation and apoptosis. .

Taken together, the data presented here suggest that endogenous GHRH acts as a growth factor through activation of MAPK/ERK (in a ras and/or raf dependent fashion). At the same time, arguing backward from the effects of antagonism, the data suggest an anti-apoptotic action of GHRH through suppression of p38 activation of a caspase cascade (9>3>2) and consequent inhibition of Bcl-2 cleavage. Activation of the Jnk pathway may antagonize the effects of GHRH on the p38 pathway. Our tentative understanding of this pathway is illustrated in Figure 11.

Figure 11: Proposed pathway for growth promoting and anti-apoptotic actions of endogenous GHRH in MDA231 cells.

Task 3: **To define the sequences of GHRH and GHRH receptor (GHRHr) promoters responsible for expression of these genes in breast cancer cells. (Months 24-36)**

Work is now beginning on this task.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that, under appropriate culture conditions, GHRH promotes MAPK phosphorylation/activation in MDA231 cells, as previously seen in pituitary somatotrophs.

- Demonstration that disruption of endogenous GHRH action in MDA231 breast cancer cells with the GHRH antagonists, GHRHa or PRL2140, results in:
  - transient activation of P38 kinase
  - transient activation of caspase 3
  - loss of Bcl-2 expression following activation of caspase 3

- Demonstration that inhibition of GHRHa activation of caspase 2,3 and 9 results in prevention of GHRHa induced cell loss.

REPORTABLE OUTCOMES

Zeitler P, Siriwardana G. Antagonism of endogenous growth hormone-releasing hormone leads to reduced proliferation and apoptosis in MDA 231 breast cancer cells. Endocrine, *In press*, July 2002. (Included in Appendix)

Zeitler P, Siriwardana G. Antagonism of endogenous growth hormone releasing hormone promotes apoptosis in MDA231 breast cancer cells: Activation of p38 and the caspase pathway. In preparation.

CONCLUSIONS

The data presented here expand our understanding of the pathway by which the antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. The studies completed in the past year now implicate specific members of the caspase family in a p38-induced cascade leading to suppression of Bcl-2 and apoptosis. Furthermore, the results begin to allow us to develop a preliminary and testable model for GHRH biology in breast neoplasia, presented in Figure 11.

The emerging picture of the pathway by which GHRH promotes growth and inhibits apoptosis in breast cancer cell lines furthers our understanding of the previously demonstrated actions of GHRH antagonists to inhibit breast cancer growth in vitro and in vivo. More importantly, this understanding begins to suggest ways in which GHRH antagonists might fit into therapeutic regimens, as pro-apoptotic agents in their own right or as adjuvant agents supporting the action of traditional anti-neoplastics. For example, overexpression of Bcl-2 may be related to drug resistance in many tumors and is an indicator of poor prognosis[34-39]. Since GHRH and/or its receptor have been reported in many cancers[17, 40-41], the ability of GHRH antagonists to promote Bcl-2 cleavage could provide a novel approach to drug resistance in cancers that express the GHRH receptor.

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Antagonism of Endogenous Growth Hormone–Releasing Hormone Leads to Reduced Proliferation and Apoptosis in MDA231 Breast Cancer Cells

Philip Zeitler and Gamini Siriwardana

Division of Endocrinology, Department of Pediatrics, University of Colorado Health Science Center and The Children's Hospital, Denver, CO

AU: shorten abstract to no more than 200 words

GHRH, in addition to stimulating the release of growth hormone (GH) from the pituitary, acts as a trophic factor for pituitary somatotrophs. Growth hormone–releasing hormone (GHRH) is also expressed outside of the hypothalamic/pituitary axis—in the gonads, gastrointestinal tract, pancreas, thymus, and lymphocytes, as well as in tumors of the pancreas, lung, central nervous system, and breast. However, the physiologic role of extrahypothalamic GHRH is unknown. Since GHRH has mitogenic effects in some extrapituitary sites, we examined the hypothesis that GHRH functions as an endogenous autocrine/paracrine growth factor in neoplastic breast tissue. MDA231 cells were grown under standard conditions and GHRH receptor expression was demonstrated by polymerase chain reaction amplification. The effect of disrupting endogenous GHRH on cell growth and apoptosis was examined through the use of a competitive GHRH antagonist, [*N*-acetyl-Tyr1, D-Arg2] fragment 1–29Amide (GHRHa) (1–3 µ*M*). Cell proliferation was determined by direct cell counting and tritiated thymidine incorporation. Apoptosis was analyzed by examination of DNA laddering and nuclear condensation. Exposure of MDA231 cells to GHRHa resulted in a dose-dependent, transient, and reversible decrease in cell number and proliferation rate. Furthermore, GHRHa resulted in a transient and reversible decrease in tritiated thymidine uptake, indicating decreased cellular proliferation rate. Conversely, exposure of MDA231 cells to GHRHa led to a marked and dose-dependent increase in both DNA laddering and nuclear condensation, implying the promotion of apoptosis. These results indicate that disruption of endogenous GHRH action in MDA231 cells results in both decreased cellular proliferation and increased apoptosis. Taken together, the findings suggest that endogenous GHRH acts as an autocrine/paracrine factor in the regulation of growth of at least some breast cancer cell types.

Key Words: Growth hormone–releasing hormone; neoplasia, breast; growth factor; apoptosis; autocrine/paracrine growth control.
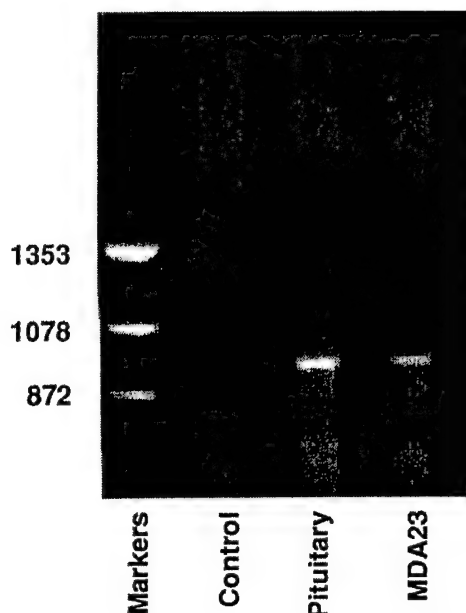
Introduction

The hypothalamic neuropeptide growth hormone–releasing hormone (GHRH) stimulates growth hormone (GH) synthesis and secretion from the pituitary and is a critical trophic factor promoting development and proliferation of pituitary somatotrophs *(1–8)*. GHRH is also expressed in a limited set of other tissues, including lymphocytes, placenta *(9,10)*, gut *(11)*, kidney *(12)*, thymus *(13)*, and testis *(10,14)*, where it is assumed to play an autocrine/paracrine role. However, the physiology of extrahypothalamic GHRH has not been well studied, although mitogenic activity has been reported in lymphocytes and testicular germ cells *(15–17)*, suggesting that a trophic role for GHRH may not be unique to the pituitary somatotroph. Recently, we *(18)* and others *(19)* have demonstrated that GHRH activates the mitogen-activated protein kinase (MAPK) pathway, as well as cellular proliferation in somatotroph cell lines, providing a potential signaling framework for mitogenic actions.

GHRH is also known to be expressed in tumors of the central nervous system, lungs, and gastrointestinal (GI) tract *(20)*. Indeed, GHRH was originally isolated from pancreatic tumors, and ectopic secretion of GHRH is a well-described cause of acromegaly. More recently, expression has been demonstrated in tumors of the breast *(21)*, prostate *(22)*, ovary, and endometrium *(23–25)*. Furthermore, GHRH receptor antagonists (GHRHa) have been reported to have antitumorigenic activity in a variety of transformed human cell lines, including GI tract, renal, prostate, ovarian, and breast *(26–31)*. However, the mechanism of action of these antagonists to inhibit tumor growth, as well as the underlying role of GHRH itself in these tumors, remains unclear.

To study GHRH biology in extrahypothalamic tumors, we were interested in establishing an in vitro model amenable to molecular dissection. Since breast cancer cell lines
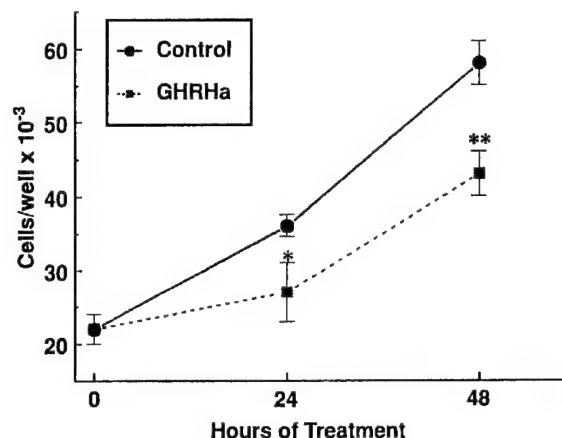
Fig. 1. RT-PCR amplification of total RNA from a human pituitary tumor and MDA231 cells. RT-PCR amplification (35 cycles) was performed using 1 µg of total RNA prepared from MDA231 cells and a human pituitary tumor and primers designed to amplify a 953-bp segment of the hGHRH receptor as described in Materials and Methods. Cloning and direct sequencing confirmed the identity of the transcript as hGHRH receptor mRNA.
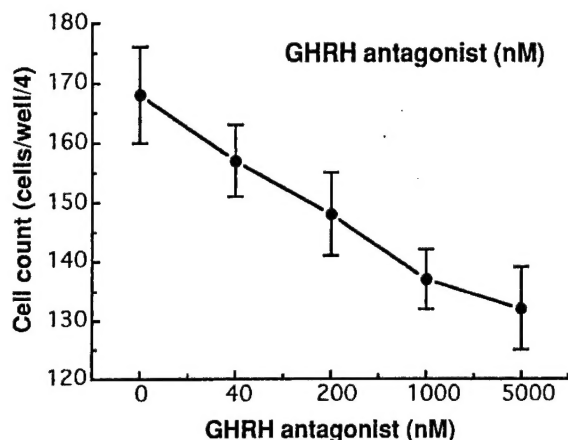

Fig. 2. Effect of GHRHa on MDA231 cell counts in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in Dulbecco's modified Eagle's medium (DMEM)/2% fetal calf serum (FCS). The medium was replaced with DMEM without serum, and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. In all experiments, control cells were treated with the same final concentration of vehicle alone. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 µL of trypsin and resuspended in 150 µL of PBS. Four 0.1-µL samples of each well were counted by hemocytometer, with eight replicates per treatment. Values represent the mean ± SEM; $n = 8$ replicates at each time point for each treatment. $*p < 0.05$; $**p < 0.01$.

express endogenous GHRH *(21,32)*, we first demonstrated that these cell lines also express the GHRH receptor and then examined the effect of disrupting the autocrine/paracrine actions of GHRH on proliferation and apoptosis of these cells using a GHRH receptor antagonist.

Results

As shown in Fig. 1, reverse transcriptase polymerase chain reaction (RT-PCR) of total RNA from MDA231 cells using hGHRHr-specific primers detected the presence of a 953-bp transcript identical in size to that present in human pituitary tissue. No transcript was present in amplifications lacking input RNA (control lane). Cloning and direct sequencing confirmed the identity of the transcript as full-length hGHRH receptor mRNA. Western blot analysis confirmed previous reports of immunoreactive GHRH in extracts from MDA231 cells *(32)*.

The effect on cell growth of disruption of endogenous GHRH signaling was examined using the hGHRH antagonist, [*N*-acetyl-Tyr1, D-Arg2] fragment 1-29Amide (Sigma, St. Louis, MO). As shown in a representative experiment (Fig. 2), a single treatment of MDA231 cells with 3 µM antagonist resulted in an approx 25% decrease in cell number after 24 h. Subsequently, cell numbers increased in parallel with control cells, indicating that the effect is transient and

reversible. A second treatment after 24 h led to an additional 24 h of inhibition of the increase in cell number. This inhibition by GHRHa was also dose dependent. As shown in a representative experiment (Fig. 3), exposure for 24 h to GHRHa resulted in decreases in cell number ranging from 6% at 40 nM to 25% at 5 µM. Higher doses did not lead to decreases >25%.
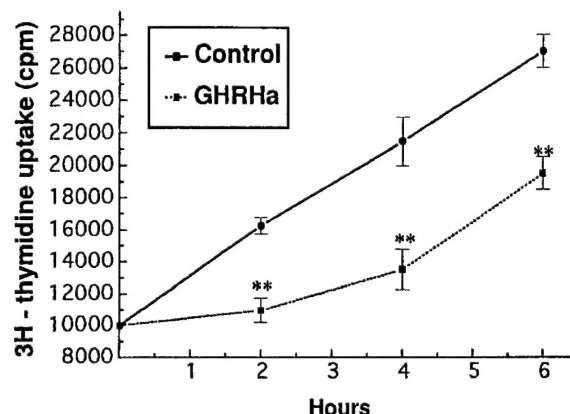
To determine the mechanism responsible for the reduction in cell number caused by exposure to GHRHa, the effect of GHRHa on measures of cellular proliferation and mitosis was examined. As shown in a representative experiment (Fig. 4), exposure of MDA231 cells to a single dose of 3 µM GHRHa resulted in a rapid and transient decline in thymidine uptake followed by uptake parallel to control cells. This change in thymidine uptake indicates a decrease in DNA synthesis and suggests a decrease in cellular proliferation. When cells were exposed to a second dose of GHRHa after 4 h, tritiated thymidine uptake was inhibited for an additional 4 h followed by recovery and uptake parallel to control cells.

To evaluate whether antagonism of endogenous GHRH also decreased cell counts through promotion of apoptosis, the effect of GHRHa on apoptosis was determined using two independent techniques. As shown in Fig. 5, exposure of MDA231 cells to 3 µM GHRHa led to a marked increase in DNA laddering compared to vehicle alone. In addition,

Fig. 3. Dose-dependent effect of GHRHa on MDA231 cell counts in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in DMEM/2% FCS. The medium was replaced with DMEM without serum, and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. In all experiments, control cells were treated with the same final concentration of vehicle alone. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 μL of trypsin and resuspended in 150 μL of PBS. Four 0.1-μL samples of each well were counted by hemocytometer, with eight replicates per treatment. Values represent the mean ± SEM; $n = 8$ replicates at each time point for each treatment.

Fig. 4. Effect of GHRHa on ^{3}H-thymidine uptake by MDA231 cells in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in DMEM/2% FCS. The medium was replaced with DMEM without serum and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS. Four hours after GHRHa treatment, 0.8 μL of ^{3}H-thymidine was added to each well. At the indicated times, cells were washed thrice with 150 μL of phosphate-buffered saline (PBS), followed by 25 μL of 10% trichloroacetic acid (TCA). After 5 min, 100 μL of 0.1 M NaOH was added followed by 27.5 μL of 0.1M HCl. The entire content of the well was transferred to scintillation counter tubes and counted for 10 min. Values represent the mean ± SEM; $n = 8$ replicates at each time point for each treatment. **$p < 0.01$.

exposure of MDA231 cells to 3 μM GHRHa for 24 h increased the frequency of appearance of condensed nuclei after staining with Hoechst dye. To quantify this increase, the number of condensed nuclei present in a ×100 field (four fields per slide, eight slides per treatment) was counted. As shown in Fig. 6, after 24 h in the presence of 3 μM GHRHa, the frequency of apoptotic cells increased by 60–75% in independent experiments. However, even after the increase in apoptotic frequency following GHRHa treatment, the overall rate of apoptosis remained limited, with approx 5% of cells in a field of 2000 demonstrating signs of apoptosis at 24 h, a rate consistent with the decrease in cell numbers at 24 h.
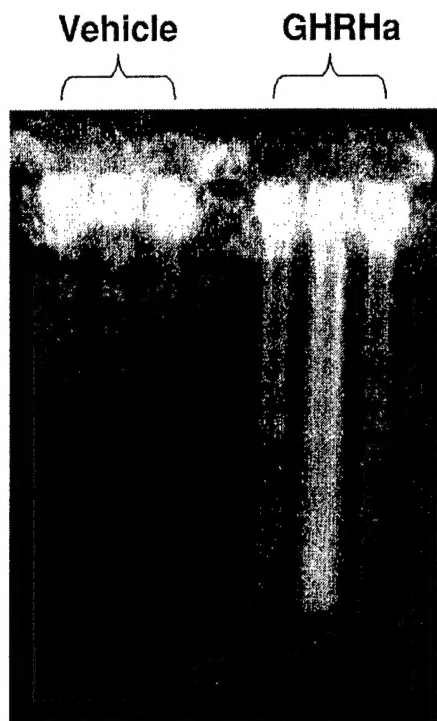
Discussion

The data presented here indicate that antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. The effect of GHRH antagonist is dose-dependent, transient, and reversible. These results are consistent with previous demonstrations of an inhibitory effect of GHRH antagonists on a variety of reproductive and GI tract tumors, extending these observations to provide initial information regarding the mechanism of the effect of GHRH antagonists.

The decrease in cell numbers seen in the MDA231 cells exposed to GHRH antagonist is unlikely to be owing to nonspecific toxicity. The effects of the antagonist are transient and completely reversible, suggesting a physiologic rather than catastrophic event. Furthermore, the effect of the antagonist on cellular proliferation is relatively modest even at maximal doses, suggesting that the effect is limited to certain cells, perhaps in a particular physiologic state or position in the cell cycle, rather than a generalized toxic effect on all cells. Finally, the decrease in cell number is associated with DNA laddering and nuclear condensation, features characteristic of apoptosis rather than nonspecific cell death.
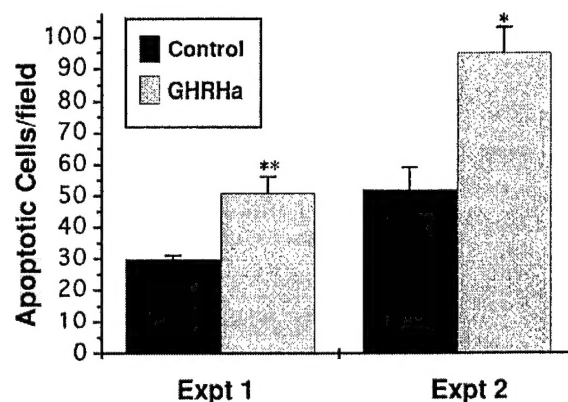
The implications of changes in thymidine uptake are arguable. On the one hand, a decrease in uptake may reflect decreased rates of DNA synthesis (i.e., reduced mitosis). Alternatively, decreased uptake may indicate reduced rates of DNA repair processes. However, in the current experiments, the association of decreased thymidine uptake with the subsequent reduction in cell number strongly suggests that exposure to GHRH antagonist is promoting a decrease in the rate of mitosis and cell proliferation. Furthermore, while it is conceivable that the decrease in thymidine uptake reflects the loss of DNA synthesis by cells undergoing apoptosis, the degree of reduced cell number appears to exceed what can be accounted for by apoptosis alone, implying that

Fig. 5. Effect of GHRHa on DNA laddering in MDA231 cells. MDA231 cells were grown as described in 3.5-cm plates overnight. Following treatment with GHRHa as described, the volume of medium was increased to 3 mL. Cells were harvested 12 h after GHRHa treatment, pelleted by centrifugation, and washed once in PBS. Cells were lysed in 20 μL of lysis buffer (50 mM Tris, pH 8.0; 10 mM EDTA; 0.5% sodium dodecyl sulfate [SDS]; 0.5 mg/mL of proteinase K) and heated to 50°C for 1 h. The mixture was then heated to 90°C for 3 min to deactivate the proteinase K, treated with 10 μL of RNase A to a final concentration of 0.5 μg/mL in TE, and heated to 50°C for 1 h. Samples were separated by electrophoresis through 2% agarose with ethidium bromide and visualized (Alphaimager). Each lane represents the total DNA sample obtained from a single replicate, with three replicates per treatment.

Fig. 6. Effect of GHRHa on nuclear condensation of MDA231 cells in vitro. Cells were grown as described in 100 μL of DMEM/2% FCS on chamber slides overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Twenty-four hours after GHRHa treatment, cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 10 min, followed by 70% EtOH in glycine buffer for 10 min at –20°C. Cells were then washed in PBS, incubated with Hoechst dye (8 μg/mL) for 15 min at room temperature, and rinsed three times in PBS. Slides were then masked and cells visualized by fluorescent microscopy and apoptotic cells counted (four fields per slide, eight slides per treatment). Values represent the mean ± SEM in two independent experiments. *$p < 0.05$; **$p < 0.01$.

at least a portion of the cell number reduction is a consequence of reduced cellular proliferation.

The concept of GHRH as a promoter of cellular proliferation is not in itself novel. Within the hypothalamic pituitary axis, extensive evidence supports the role of GHRH in the development and proliferation of GH-secreting somatotrophs. GHRH stimulates the expression of both the GH gene and c-*fos*, and enhances somatotroph proliferation in vitro *(4–6,18,33–35)*. Long-term exposure to GHRH in vivo results in somatotroph hyperplasia in animals *(36,37)* and humans *(38,39)*. Conversely, rats in which GHRH action is transiently impaired during the neonatal period have reduced pituitary size and somatotroph cell number *(7,40–42)*. Similarly, resistance to GHRH action, as in the *lit* mouse *(43, 44)* or *dw* rat *(2)*, or congenital absence of GHRH, as in the GSH-1 knockout mouse *(1)*, is associated with marked somatotroph hypoplasia.

However, the mechanism by which GHRH promotes cellular proliferation is unclear. In pituitary somatotrophs, analogs of cyclic adenosine monophosphate (cAMP) and somatotroph-targeted expression of cholera toxin in transgenic animals induce cellular proliferation in culture *(35,45)*. Conversely, GH-promoter driven overexpression of dominant negative CREB leads to somatotroph hypoplasia *(8)*, a finding interpreted to indicate that inhibition of the transcriptional effects of cAMP prevents the genomic and proliferative effects of GHRH. Recently, we *(18)* and others *(19)* have demonstrated that GHRH activates the MAPK pathway, as well as cellular proliferation in somatotroph cell lines. Furthermore, proliferation in response to GHRH was prevented by agents that prevent activation of MAPK, strongly implying that GHRH promotes proliferation, at least in part, through activation of the MAPK pathway.

The question of which receptor is transducing extrahypothalamic actions of GHRH has been somewhat controversial. In some cases, attempts at identifying the GHRH receptor in GHRH antagonist–responsive tumors has been unsuccessful *(21,29,46)*, and it has been suggested that GHRH may be acting through related vasoactive intestinal peptide or PACAP receptors *(47,48)*. However, in the case of MDA231 breast cancer cells, the presence of GHRH receptor mRNA suggests that the actions of GHRH on cellular proliferation are likely mediated by the GHRH receptor itself.

In summary, the results of these experiments indicate that exposure of MDA231 breast cancer cells to a GHRH receptor antagonist in vitro results in reduced cell numbers. Furthermore, the experiments provide evidence that the decrease in cell number reflects both decreased cellular proliferation, as indicated by decreased tritiated thymidine uptake, and increased cellular apoptosis, as indicated by increased DNA laddering and nuclear condensation. Taken together, the data suggest that disruption of endogenous GHRH receptor signaling results in disruption of normal MDA231 cellular dynamics, leading to decreased proliferation and survival of the breast cancer cells. By extension, these results imply that endogenous GHRH supports MDA231 cell proliferation and inhibits apoptotic pathways.

Materials and Methods

Cell Culture

MDA231 cells, originally obtained from American Type Culture Collection, were grown to confluency under standard conditions in DMEM supplemented with 10% FCS. Prior to experiments, the cells were removed following treatment with PBS/2% EDTA; plated at 8000/cm in 96-, 24-, or 6-well plates; and allowed to attach overnight in DMEM/2% FCS. For treatments, the medium was replaced with DMEM without serum and treatments applied for 1 h, following which medium was brought to 2% FCS and maintained until harvest. The competitive GHRH antagonist GHRHa was dissolved in 2% acetic acid/1% insulin-free bovine serum albumin to a stock concentration of 1 mM. In all experiments, control cells were treated with the same final concentration of vehicle alone.

RT-PCR Amplification

Total RNA was prepared from MDA231 cells and a human pituitary tumor using commercial reagents. RT-PCR amplification (35 cycles) was performed using 1 μg of total RNA from each tissue and primers designed to amplify a 953-bp segment of the hGHRH receptor as previously described (49).

Cell Counts

Cells were grown as described in 100 μL of DMEM/2% FCS in a 96-well plate overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 μL of trypsin and resuspended in 150 μL of PBS. Four 0.1-μL samples of each well were counted by hemocytometer, with eight replicates per treatment.

Tritiated Thymidine Uptake

Cells were grown as described in 100 μL of DMEM/2% FCS in a 96-well plate overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Four hours after GHRHa treatment, 0.8 μL of ^{3}H-thymidine was added to each well. At the indicated times, cells were washed thrice with 150 μL of PBS, followed by 25 μL of 10% TCA. After 5 min, 100 μL of 0.1 M NaOH was added followed by 27.5 μL of 0.1M HCl. The entire content of the well was transferred to scintillation counter tubes and counted for 10 min. Each treatment was examined in eight replicates.

DNA Ladder

Cells were grown as described in 3.5-cm plates overnight. Following treatment with GHRHa as described, the volume of medium was increased to 3 mL. Cells were harvested 12 h after GHRHa treatment, pelleted by centrifugation, and washed once in PBS. Cells were lysed in 20 μL of lysis buffer (50 mM Tris, pH 8.0; 10 mM EDTA; 0.5% SDS: 0.5 mg/mL of proteinase K) and heated to 50°C for 1 h. The mixture was then heated to 90°C for 3 min to deactivate the proteinase K, treated with 10 μL of RNase A to a final concentration of 0.5 μg/mL in TE, and heated to 50°C for 1 h. Samples were separated by electrophoresis through 2% agarose with ethidium bromide and visualized (Alphaimager).

Hoechst Staining

Cells were grown as described in 100 μL of DMEM/2% FCS on chamber slides overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Twenty-four hours after GHRHa treatment, cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 10 min, followed by 70% EtOH in glycine buffer for 10 min at −20°C. Cells were then washed in PBS, incubated with Hoechst dye (8 μg/mL) for 15 min at room temperature, and rinsed three times in PBS. Slides were masked so that the reader would be unaware of the treatment exposure, cells were visualized by fluorescent microscopy, and apoptotic cells were counted (four fields per slide, eight slides per treatment).

Statistical Analysis

Where indicated, data were analyzed by one-way analysis of variance followed by post-hoc analysis with the Neuman-Keuls test.

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